Comparison of the Phosfinity ChainQuant kit, polyacrylamide gel electrophoresis, and other methods for the determination of the average polyphosphate chain length

Dr. Jonas J. Christ (Aminoverse B.V., Nuth, Netherlands; contact: phosfinity@aminoverse.com)
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Summary

Measuring results of five different methods for the quantification of the average polyphosphate (polyP) chain length were compared. Among them was the Phosfinity ChainQuant kit from Aminoverse B.V. On average, the average polyP chain length deviated from Phosfinity ChainQuant by -4 % (^{31}P nuclear magnetic resonance, NMR), 14 % (end group titration), and 26 % (an alternative enzyme assay) with several tested polyPs. Hence, acceptable to good agreement between Phosfinity ChainQuant, ^{31}P NMR, an alternative enzyme assay, and end group titration was found.

The results between PAGE (polyacrylamide gel electrophoresis) and the other methods differed vastly. E.g., the average polyP chain length of one polyP was quantified to be 17 P-subunits (^{31}P NMR), 19.9 P-subunits (Phosfinity ChainQuant), 21.2 P-subunits (end group titration), 25.7 P-subunits (alternative enzyme assay), and 90 P-subunits (PAGE). On average, the average polyP chain length differed between Phosfinity ChainQuant and PAGE by 348 % with several tested polyPs.

Reasons for the large deviation between PAGE and the other methods could be: A) Short chain polyP (< 15 P-subunits) is not detected in standard PAGE. B) DAPI and toluidine blue bind stronger to longer polyP chains. Thus, short chain polyP does not appear as bright on the gel as the longer chains. C) Shorter polyP chains diffuse quicker out of the polyacrylamide gel during staining procedures. D) The quantitative analysis of PAGE gels requires picture analysis software, which possibly introduces further errors. E) Standard PAGE relies on size markers in comparison to absolute methods, such as ^{31}P NMR. The results suggest that PAGE is not an optimal tool for the quantification of the average polyP chain length, but rather suited for the determination of the polyP chain length distribution.

The study confirmed that Phosfinity ChainQuant, ^{31}P NMR, an alternative enzyme assay, and end group titration are suitable methods for the determination of the average polyP chain length. However, ^{31}P NMR requires an expensive NMR machine and takes about 30 min per sample. End group titration is limited to pure polyP. The alternative enzyme assay is limited to polyP with a chain length of ca. < 50 P-subunits. Phosfinity ChainQuant can measure all polyP chain lengths, is affordable, requires only a plate reader, and tolerates contaminating substances. Thus, Phosfinity ChainQuant is the ideal tool for the polyP researcher.

Introduction

Inorganic polyphosphate (polyP) is the polymer of orthophosphate and can be found in many living organisms. Many methods for the quantification of the average polyP chain length have been published (see Christ et al. (2020) for a review). The goal of this study was to compare the Phosfinity ChainQuant kit, polyacrylamide gel electrophoresis, and other methods for the quantification of the average polyP chain length.

Methods

Polyphosphates

Both chemically and biologically produced polyPs were tested. See the cited literature for the origin of the polyPs.

Phosfinity ChainQuant kit

Aminoverse B.V. has released a kit for the determination of the average polyP chain length by enzyme assay (Aminoverse, 2023). The kit utilizes enzymes coupled with colorimetric and fluorometric quantification with a plate reader. The kit mechanics are explained in detail in Christ et al. (2019). Briefly, the average polyP chain length is measured as the quotient of the total polyP concentration (i.e., polyP monomers per liter) and the polyP chain concentration (i.e., mole polyP chains per liter). The total polyP concentration is determined by enzymatic polyP hydrolysis with *Saccharomyces cerevisiae* exopolyphosphatase 1 (PPX; $polyP_n$ → pyrophosphate + *n* - 2 phosphate) and *S. cerevisiae* inorganic pyrophosphatase 1 (IPP; pyrophosphate → 2 phosphate), followed by colorimetric phosphate quantification with ascorbate-molybdate. The polyP chain concentration is determined by treatment with PPX ($polyP_n$ → pyrophosphate + *n* - 2 phosphate), ATP sulfurylase (pyrophosphate + adenosine 5'-phosphosulfate → ATP + sulfate), hexokinase (ATP + glucose → glucose 6-phosphate + ADP), and glucose 6-phosphate dehydrogenase (glucose 6-phosphate + $NADP^+$ → 6-phosphogluconolactone and NADPH), followed by fluorometric NADPH quantification.

PAGE

PolyP is separated in a polyacrylamide gel by electric current. Subsequent staining can be done with toluidine blue, or DAPI (negative staining). Semi-quantitative analysis is done by picture analysis software, where the intensity of a polyP "smear" is analyzed and plotted against a molecular weight nucleic acid standard. Note, that high strength polyacrylamide can be used to count individual polyP bands, each representing one polyP chain length, up to ca. 50 P-subunits.

In this study, the average polyP chain length was determined by DAPI staining of the polyacrylamide gel and subsequent picture analysis. The staining intensity was plotted against the molecular weight, which was determined from nucleic acid molecular weight markers that were previously correlated to polyP molecular weights (Smith et al., 2018). See https://www.kerafast.com/Images/p100QC.jpg for an example of such a plot.

^{31}P nuclear magnetic resonance spectroscopy (^{31}P NMR)

Nuclear magnetic resonance signals appear for the terminal P-groups (PP1), the penultimate (PP2, PP3), and the core P-groups (PP4) of the polyP. The average polyP chain length is calculated absolutely (i.e., without standards) from the ratio of those signals.

End group titration

The method is based on the fact that each end group of a linear polyP chain possesses a hydroxyl group with a neutral pK_a value. The concentration of the end groups is titrated in an acid–base titration. The polyP chain concentration is calculated by dividing the end group concentration by two (two end groups per polyP chain). The polyP sample is also subjected to a total P determination, as described above for the Phosfinity ChainQuant Kit. The average polyP chain length is calculated by dividing the total P concentration through the chain concentration.

Enzyme assay 2

The assay utilizes the same principle as the Phosfinity Chain Quant kit, i.e. dividing the total polyP concentration through the polyP chain concentration to obtain the average polyP chain length (Christ and Blank, 2018). Briefly, the total polyP concentration is measured as in the Phosfinity Chain Quant Kit, i.e by hydrolysis with PPX and IPP ($polyP_n$ → *n* phosphate), followed by colorimetric phosphate quantification. The chain concentration, however, is quantified as follows: the sample is hydrolyzed by PPX ($polyP_n$ → $polyP_2$ + n - 2 phosphate), followed by phosphate quantification. The released $polyP_2$ is measured by subtracting the total polyP concentration (PPX plus IPP digest) from the phosphate concentration obtained just by hydrolysis with PPX, and indicates the polyP chain concentration.

Results

Seven chemically produced polyPs with an average chain length of 2 to 274 P-subunits were analyzed with Phosfinity ChainQuant and ^{31}P NMR (Table 1). The deviation between method ranged from -19 to 8 %. Higher cyclic polyP contents (measured by ^{31}P NMR) seemed to increase deviation between both methods.

Table 1. Average chain length determination of seven chemically produced polyPs with the Phosfinity ChainQuant kit and ^{31}P NMR (Christ et al., 2019)

	mean chain length ± standard error of the mean			
polyP	enzyme assay	^{31}P NMR	deviation between methods [%]	cyclic polyP [relative to linear polyphosphate][a]
$polyP_2$	1.9 ± 0.0	2.0	4	0.00
$polyP_3$	2.9 ± 0.0	2.9	3	0.02
m. 155	6.0 ± 0.2	5.6	−6	0.83
Budit 4	20.3 ± 0.1	17.0	−16	6.81
p700	53.1 ± 1.1	50.4	−5	1.53
p100	54.2 ± 0.8	44.1	−19	6.35
m. 385	274.3 ± 5.3	297.3	8	0.29

[a]Determined with ^{31}P NMR. [b]For the enzyme assay, the experimental design was 2 × 7 × 3 (number of independent experiments done on separate days × number of tested polyP's × number of replicate measurements). For ^{31}P NMR, the experimental design was 1 × 7 × 1. Abbreviation: m., mode.

Seven chemically produced polyPs with an average chain length of 2.9 to ca. 25 P-subunits were analyzed with Phosfinity ChainQuant, the alternative enzyme assay, and end group titration (Table 2). The deviation between Phosfinity ChainQuant and the alternative enzyme assay ranged from 7 to 39 %. The deviation between Phosfinity ChainQuant and end group titration ranged from 4 to 22 %. The deviation between the alternative enzyme assay and end group titration ranged from -18 to 8 %.

Table 2. Average chain length determination of five chemically produced polyPs with the Phosfinity ChainQuant kit, an alternative enzyme assay and end group titration (Christ and Blank, 2018; Christ et al., 2019; Christ et al., 2020 b)

Sample	Phosfinity Chain Quant	Alternative Enzyme Assay[a]	End group titration[a]	Deviation…		
				Phosfinity Chain Quant to the alternative enzyme assay	Phosfinity Chain Quant to end group titration	Alternative enzyme assay to end group titration
Triphosphate	2.9 ± 0.0[b]	3.1 ± 0.0	n.d.	7 %		
Budit 9	n.d.	3.8 ± 0.0	4.1 ± 0.0			8 %
PolyP from Sigma	n.d.	11.8 ± 0.1	10.8 ± 0.0			-8 %
Budit 7	10.0[c]	12.6 ± 0.3	11.9 ± 0.0	26 %	19 %	-6 %
Budit 4	20.3 ± 0.1[b] / 19.4 [d]	25.7 ± 2.3	21.2 ± 0.1	27 % / 32 %	4 % / 9 %	-18 %
PolyP from Roth	20.1[d]	27.9 ± 2.7	24.6 ± 0.1	39 %	22 %	-12 %

[a] Data from Christ et al. (2018)
[b] Data from Christ et al. (2019)
[c] Data from Aminoverse laboratory
[d] Data from Christ et al. (2020b)
Abbreviation: n.d., not determined

The average chain length of four biologically produced polyPs and three chemically produced polyPs were quantified with Phosfinity ChainQuant and PAGE (Table 3, Figure 1). Note that the "p100" polyP from Table 3/Figure 1 is not the same as the "p100" from Table 1. The deviation between both methods ranges from 188 to 573 %.

Table 3. Average chain length determination of four biologically produced polyPs and three chemically produced polyPs with the Phosfinity Chain Quant kit and PAGE (Christ et al., 2020b)

Sample	Biologically produced polyP				Chemically produced polyP		
	Sodium inter-mediate chain length	Sodium short chain length	Potassium inter-mediate chain length	Potassium short chain length	Budit 4	Roth	P100
PAGE lane Fig. 1	2, 3	4, 5	6, 7	8, 9	10	11	12
Average chain length by Phosfinity ChainQuant	42.3 ± 2.2	11.3 ± 0.2	32.6 ± 0.6	12.3 ± 0.2	19.2	20.1	42.0
Average chain length by PAGE	122 ± 2	76 ± 0	110 ± 1	72 ± 1	90	85	152
Deviation	+188 %	+573 %	+237 %	+485 %	+369 %	+423 %	+262 %

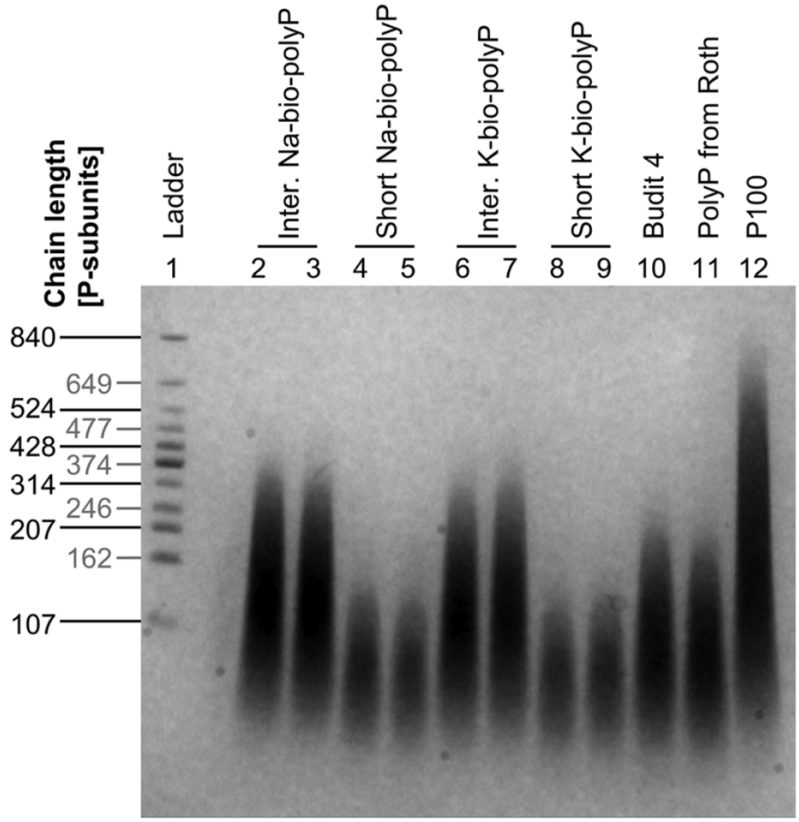

Figure 1. PAGE analysis (DAPI negative staining) of biologically produced polyP (lanes 2 to 9) and chemically produced polyP (lanes 10 to 12). Lanes 2 and 3, 4 and 5, 6 and 7, and 8 and 9 are replicated analyses of different production batches. (Christ et al., 2020b)

The data from Tables 1 to 3 is summarized in Figure 2, where the deviation between Phosfinity ChainQuant and PAGE, ^{31}P NMR, enzyme assay 2, and end group titration for the quantification of the average polyP chain length is shown. Minimum and maximum deviations are depicted by the error bars in Figure 2. The number of tested polyPs is indicated by *i*. On average, the average polyP chain length (red diamonds) deviated from Phosfinity ChainQuant by -4 % (^{31}P NMR), 26 % (enzyme assay 2), and 14 % (end group titration). The deviation between Phosfinity ChainQuant and PAGE was much greater (348 %).

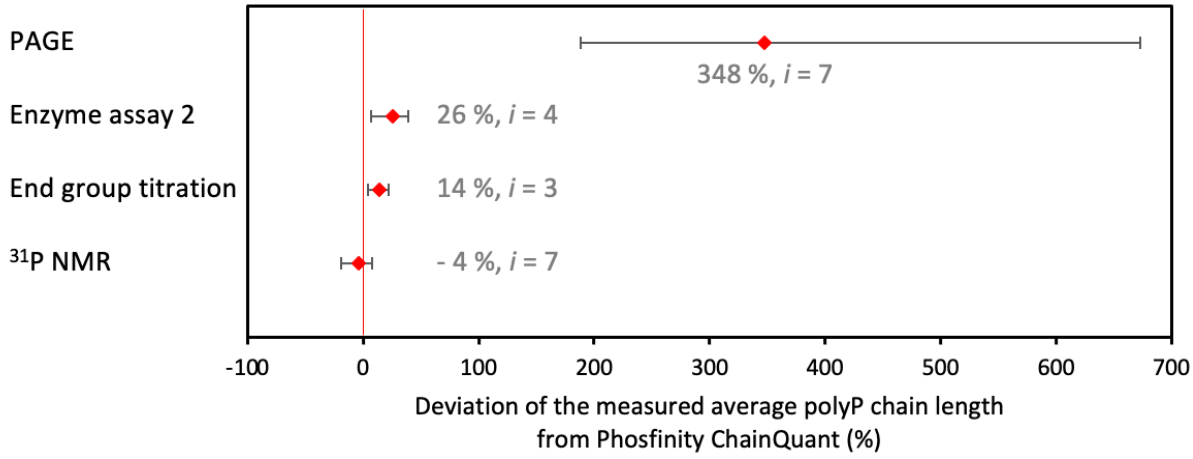

Figure 2. Deviation between Phosfinity ChainQuant and PAGE, ^{31}P NMR, an alternative enzyme assay, and end group titration for the quantification of the average polyP chain length (Data taken from Tables 1 to 3)

Red diamonds represent the mean values. Error bars indicate minimum and maximum values. *i* gives the number of tested polyPs.

Discussion

^{31}P NMR can be considered the gold standard for the quantification of the average polyP chain length in academic research. End group titration is the widely accepted standard for companies that chemically produce polyP. Good agreement between Phosfinity ChainQuant and ^{31}P NMR (on average: -4 % deviation), and between Phosfinity ChainQuant and end group titration (on average: 14 % deviation) was found.

The alternative enzyme assay utilizes no complex enzyme cascade for the quantification of the polyP chain concentration as Phosfinity ChainQuant does. Enzyme assay 2, however, is limited to polyP with a chain length of shorter than 30-40 P-subunits. Phosfinity ChainQuant, on the other hand, can analyze all polyP chain lengths. Acceptable agreement between Phosfinity ChainQuant and an alternative enzyme assay was found (on average: 26 % deviation).

PAGE results were obtained from an independent laboratory (Jim Morrissey lab). The results between PAGE and Phosfinity ChainQuant differed vastly (on average: 348 % deviation). Many factors may contribute to the deviation:

A) Short chain polyP (< 15 P-subunits) is not detected in standard PAGE with toluidine blue or DAPI staining. If the sample contains plenty of this size fraction results are skewed.
B) DAPI and toluidine blue bind stronger to longer polyP chains. Thus, short chain polyP does not appear as bright on the gel as the longer chains.
   Note: In high strength polyacrylamide gels individual polyP bands can be counted up to ca. 50 P-subunits. This alleviates the issue of not detecting shorter polyP chains and the dependence on size standards. However, since shorter chains do not interact well with DAPI and toluidine blue, the staining intensity is lower in comparison to longer chains. Therefore, a weak $polyP_4$ band should be interpreted as a large amount of $polyP_4$. Whereas, a strong $polyP_{100}$ band might indicate little amount of $polyP_{100}$. Without a correction of the staining intensity for the polyP chain length, a quantitative readout of the PAGE gel is not possible, at best only semi-quantitatively.
C) Shorter polyP chains diffuse quicker out of the polyacrylamide gel during staining procedures. Therefore, the amount of detected short chain polyP is artificially reduced.
D) The quantitative analysis of PAGE gels is done by picture analysis software. The staining intensity is read out over the whole length of the polyP “smear”. This appears to be a more error prone way of analysis in comparison to absolute methods, such as ^{31}P NMR, which do not require size standards.
E) Standard PAGE relies on size markers (oftentimes DNA ladders). DNA ladders need to be correlated to polyP sizes, which is one reason for unprecise results. Furthermore, relative methods tend to give less precise results in comparison to size standard independent (i.e. absolute) methods, such as ^{31}P NMR. In ^{31}P NMR, peak sizes are compared without the need for any standards.

Concluding, PAGE is not a suitable tool for the quantification of the average polyP chain length. PAGE is a good tool for the determination of the polyP chain length distribution, which is also an important parameter in polyP analysis. The average polyP chain length and the polyP chain length distribution, however, are two different parameters.

The results from Phosfinity ChainQuant, ^{31}P NMR, end group titration and an alternative enzyme assay agree to an acceptable to good degree. Therefore, it was confirmed that Phosfinity ChainQuant, ^{31}P NMR, enzyme assay 2, and end group titration are suitable methods for the determination of the average polyP chain length.

However, ^{31}P NMR requires an expensive NMR machine (ca. 500.000 Euro) and takes about 30 min per sample. On the upside, ^{31}P NMR may give you more insight into your sample as more parameters are measured (see Christ et al., 2020, for a review).

End group titration is limited to pure polyP. That is why it is the preferred method for chemical industry where usually only pure polyP is synthesized.

The alternative enzyme assay is limited to polyP with a chain length of ca. < 50 P-subunits. This is due to the fact that the polyP chain concentration is measured indirectly, i.e. by subtracting the phosphate concentration of the PPX+IPP digest from the phosphate concentration of the PPX digest. For longer polyP, this difference might only be a few µM polyP which cannot be precisely detected in colorimetric phosphate assays.

Phosfinity ChainQuant

- can measure all polyP chain lengths (tested up to 280 P-subunits due to no longer standards being available),
- is affordable (6,99 Euro / sample),
- requires only a plate reader capable of reading absorbance and fluorescence,
- measures hundreds of sample at once (microtiter plate based assay),
- is highly specific due to the used enzymes,
- requires samples in the microliter range, e.g. 200 µL of a 100 µM polyP (as monomer) solution,
- and tolerates contaminating substances (see the manuals).

Thus, Phosfinity ChainQuant is an ideal tool for the polyP researcher.

Literature

Aminoverse (2023) Homepage for the Phosfinity ChainQuant Assay, https://www.aminoverse.com/enzyme-products/phosfinity-chainquant/

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